Dendritic Cells Endocytose *Bacillus anthracis* Spores: Implications for Anthrax Pathogenesis[1]

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Phagocytosis of inhaled *Bacillus anthracis* spores and subsequent trafficking to lymph nodes are decisive events in the progression of inhalational anthrax because they initiate germination and dissemination of spores. Found in high frequency throughout the respiratory track, dendritic cells (DCs) routinely take up foreign particles and migrate to lymph nodes. However, the participation of DCs in phagocytosis and dissemination of spores has not been investigated previously. We found that human DCs readily engulfed fully pathogenic Ames and attenuated *B. anthracis* spores predominately by coiling phagocytosis. Spores provoked a loss of tissue-retaining chemokine receptors (CCR2, CCR5) with a concurrent increase in lymph node homing receptors (CCR7, CD11c) on the membrane of DCs. After spore infection, immature DCs displayed a mature phenotype ($CD83^{bright}$, $HLA\text{-}DR^{bright}$, $CD80^{bright}$, $CD86^{bright}$, $CD40^{bright}$) and enhanced costimulatory activity. Surprisingly, spores activated the MAPK cascade (ERK, p38) within 30 min and stimulated expression of several inflammatory response genes by 2 h. MAPK signaling was extinguished by 6 h infection, and there was a dramatic reduction of secreted TNF-α, IL-6, and IL-8 in the absence of DC death. This corresponded temporally with enzymatic cleavage of proximal MAPK signaling proteins (MEK-1, MEK-3, and MAP kinase kinase-4) and may indicate activity of anthrax lethal toxin. Taken together, these results suggest that *B. anthracis* may exploit DCs to facilitate infection. *The Journal of Immunology,* **2005, 174: 5545–5552.**

Inhalational anthrax, a disease that was exploited for bioterrorism (1), is most often fatal and causes lasting disabilities for survivors (2). Following inhalation, *Bacillus anthracis* spores lay latent in lung fluids until they are engulfed by phagocytic cells (3) and carried across lung tissues into the lymphatics (4). These decisive steps initiate infection by triggering germination of spores into vegetative bacilli. Intracellular bacilli quickly multiply, escape host cells, and are released into lymphatics. From there, bacilli gain access to the circulatory system (5) and are disseminated throughout the host (5). Further tissue damage is caused by edema toxin (ET)[3] and lethal toxin (LT), encoded on plasmid pX01. Fatality often results from rupture of inflamed mediastinal lymph nodes, causing secondary sepsis, edema, and multiple organ failure (6).

For decades, research has focused almost exclusively on macrophages as an anthrax Trojan horse (7) that engulfs spores and carries them out of the lungs. However, mice that were chemically depleted of macrophages and infected with spores by aerosol nevertheless experienced disease (8). Cote et al. (8) demonstrated that macrophages were not essential for initiation of infection and implicated the existence of unidentified cell populations within the lungs that may be exploited by *B. anthracis* to facilitate the disease process. In particular, the dendritic cell (DC) has qualities that make it a strong candidate for exploitation by *B. anthracis* to aid in progression of disease. DCs are found in high frequency within the airway epithelium, submucosa, lung parenchyma, and alveolar space (9). Migration of Ag-loaded DCs from these regions into draining lymph nodes is fundamental to initiating acquired immune responses. Activated DCs initiate innate immune responses via contact interactions with other leukocytes and by secretion of proinflammatory chemokines, cytokines, and lipid mediators. During maturation, DCs increase membrane expression of costimulatory proteins, thus allowing them to efficiently present Ags to cognate T lymphocytes, initiating adaptive immune responses. Therefore, DCs are regarded as a critical link between the innate and adaptive immune responses (10) in the respiratory mucosa (9). The central role of DCs in development of immunity and their ability to take up and transport particles from the lungs led us to postulate that *B. anthracis* might exploit DCs to invade the host and to evade host immune responses.

In the present study, we examined by confocal and electron microscopy the capacity for human DCs to endocytose fully pathogenic Ames and nonpathogenic *B. anthracis* spores in vitro. We determined that DCs endocytose *B. anthracis* spores primarily by coiling phagocytosis. Next, we evaluated the impact of infection and *B. anthracis* virulence factors on DC immunostimulatory activities by infecting DCs with a strain that secretes ET and LT (Sterne) and a strain that lacks the capacity to produce these toxins (difference in Ames nonresponder (ΔANR). DC immunostimulatory activities were assessed by examination of RNA and protein levels of chemokine

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[3] Abbreviations used in this paper: ET, edema toxin; ANR, Ames nonresponder; DC, dendritic cell; LT, lethal toxin; MKK, MAP kinase kinase; MOI, multiplicity of infection; RPA, RNase protection assay; SAPK, stress-activated protein kinase; TEM, transmission electron microscopy.

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14. ABSTRACT

Phagocytosis of inhaled Bacillus anthracis spores and subsequent trafficking to lymph nodes are decisive events in the progression of inhaled anthrax because they initiate germination and dissemination of spores. Found in high frequency throughout the respiratory tract, DCs routinely take up foreign particles and migrate to the lymph nodes. However, the participation of DCs in phagocytosis and dissemination of spores was not investigated previously. We found that human DCs readily engulfed fully pathogenic and attenuated B. anthracis spores. Spores provoked a loss of tissue-retaining chemokine receptors (CCR2, CCR5) with a concurrent increase in lymph node homing receptors (CCR7, CD11c) on the membrane of DCs. After spore infection, immature DCs displayed a mature phenotype (CD83-bright, HLA-DR-bright, CD80- bright, CD86-bright, CD40 bright), and enhanced co-stimulatory activity. Surprisingly, spores activated the mitogen-activated protein kinase (MAPK) cascade (ERK, p38) and stimulated expression of several inflammatory response genes. MAPK signaling was extenguished by 6 h after infection and resulted in dramatically reduced secretion of TNF-a, IL-6, and IL-8, without inducing DC death. This corresponded temporally with enzymatic cleavage of proximal MAPK signaling proteins (MEK-1, -3, and MKK-4) and may indicate activity of anthrax lethal toxin. Taken together, these results suggest that B. anthracis may exploit DCs to facilitate infection.

15. SUBJECT TERMS

Bacillus anthracis, anthrax, phagocytosis, spores, dendritic cells, chemokines

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receptors, proinflammatory cytokines, chemokines, and as well as by activation of MAPK proteins and stimulation of allogenic T cells. We show that DCs can be a receptive host for *B. anthracis* spores and bacilli, which stimulate secretion of TNF-α, IL-6, and IL-8 via activation of the MAPK pathway until virulence factors reach sufficient levels to silence MAPK signaling 6 h after infection. Infected DCs mature and develop the capacity for migration to lymph nodes. The study described in this work supports an alternative model for *B. anthracis* host invasion and dissemination based on DC endocytosis of spores.

Materials and Methods

Culture and infection of human monocyte-derived DCs

DCs were cultured from peripheral blood, as described previously (11). Briefly, peripheral blood monocytes were cultured for 4 days in RPMI 1640 supplemented with 1% MEM nonessential amino acids, 10% heat-inactivated FBS (henceforth referred to as cRPMI), 100 IU of penicillin, 100 μg/ml streptomicin, 20 ng/ml human IL-4, and 100 ng/ml human GM-CSF (PeproTech). Cultures were >97% $CD11c^+$ cells. Before all experiments, DCs were washed three times to remove antibiotics, and then resuspended in cRPMI. We initially tested a variety of multiplicity of infection (MOI) (1, 5, 10, and 50), but saw little difference in responses (of MAPK signaling, cytokine secretion, and induced cytokine/chemokine receptor RNA) other than slow responses at very low MOI and faster responses at very high MOI. In this study, an MOI of 10 was used because this is a typical MOI used in studies that investigate interactions of *B. anthracis* and the immune system. DCs were infected with an MOI of 10, unless otherwise noted.

B. anthracis *spores*

Culture, spore preparation, and engineering of GFP-Sterne and GFP-ΔANR spores were described previously (12). Briefly, plasmid pAFp8gfp was used in transfections of *B. anthracis* bacilli to produce bacilli that constitutively express GFP. pAFp8gfp contains a kanamycin resistance gene and a synthetic *B. anthracis* promoter sequence ligated upstream of *gfp*. The extrachromosomal plasmid is replicated during proliferation and segregates with progeny cells. During sporulation, GFP is incorporated inside spores (determined by immunoelectron microscopy; data not shown), giving rise to GFP spores. Spores were prepared using endotoxin-free reagents. Ames spores were provided by T. Abshire and J. Ezzel (U.S. Army Medical Research Institute of Infectious Diseases). Viable spores were heat shocked for 45 min at 65°C, centrifuged for 10 min at 16,000 $\times$ *g*, and resuspended in cRPMI immediately before infections. In some experiments, Sterne spores were inactivated by irradiation (4×10^6 rad) on ice.

Fluorescence microscopy

DCs were infected for 1 h, followed by five washes with 50 ml of cRPMI to remove non-cell associated spores. DCs were cultured in chamber slides coated with CC2 (Nalge Nunc International) to promote adherence to the glass slide and fixed in 0.1% paraformaldehyde overnight. DCs infected with Ames strain spores were fixed in 4% paraformaldehyde for 7 days. Images were collected using a Bio-Rad 2000MP confocal microscope. In some experiments, DCs were permeabilized with 0.2% Triton X-100 in PBS before the cells were stained with Texas Red-phalloidin, Hoechst stain (Molecular Probes), or Abs specific for CD11c (BD Biosciences).

Electron microscopy

For transmission electron microscopy (TEM), spore-infected DCs were fixed for 1 h with 2.5% glutaraldehyde prewarmed to 37°C, and were treated with 1% osmium tetroxide in a modified Millonig's buffer (0.1 M PBS, 0.5% dextrose, pH 7.3) (13) stained en bloc with 0.5% uranyl acetate in ethanol, dehydrated in graded ethanol and propylene oxide, and embedded in Poly/Bed 812 resin (Polysciences). Ultrathin sections were placed on 200-mesh nickel grids and stained with 5% uranyl acetate and 0.2% lead citrate.

RNase protection assay (RPA)

The RiboQuant MultiProbe RPA was performed according to manufacturer's directions (BD Biosciences) with minor modifications (14). Radiolabeled probe sets hCR5 and hCK2b were used to hybridize with total RNA purified from TRIzol-treated samples. Band density was quantitated using the UN-SCAN-IT gel automated digitizing system (Silk Scientific). The relative mRNA levels were determined by normalization of band densities for each protected probe fragment with that of L32, a housekeeping gene used as an internal loading control (15).

Mixed lymphocyte reaction

Immature DCs were treated with 100 ng/ml LPS (*Escherichia coli* O11:B4), Sterne spores, or ΔANR spores for 6 h before cultures were irradiated by 2000 rad. Different numbers of DCs (0–20,000) were cultured with 100,000 allogenic T cells purified by immunomagnetic depletion using anti-CD56, anti-CD19, anti-CD14, anti-HLA-DR Abs (BD Biosciences), and Dynabeads (Dynal Biotech). Cells were cultured in round-bottom 96-well plates in cRPMI supplemented with 10 μg/ml gentamicin to kill bacilli and spores. T cell proliferation was measured by the addition of 1 μCi/well [^{3}H]thymidine (Amersham Biosciences) for the final 18 h of 5-day culture. Liquid scintillation counting was used to measure incorporated [^{3}H]thymidine.

Cell surface and cytokine analysis

For cell surface analysis of protein expression, DCs were treated with LPS or infected with Sterne or ΔANR spores for 2 h before gentamicin was added to the culture medium. After 24 h of incubation, DCs were incubated with directly conjugated Abs for surface proteins (BD Biosciences) for 30 min before excess Ab was washed away. Then cells were fixed with 5% formalin overnight, and analyzed by flow cytometry. For cytokine analysis, DCs were treated with LPS or infected with Sterne or ΔANR spores for the times indicated. Culture supernatants were sterile filtered and analyzed for secreted proteins by cytometric bead array (human inflammation and human chemokine kits; BD Biosciences), according to the manufacturer's directions. All analyses were performed on a FACSCalibur (BD Biosciences).

Western blot

DCs were lysed in buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, protease inhibitor mixture, and phosphatase inhibitor mixture I and II (Sigma-Aldrich), which does not lyse *B. anthracis* spores or bacilli. Cellular proteins (30 μg of protein/sample) were separated by SDS-PAGE, then blotted with the appropriate Abs.

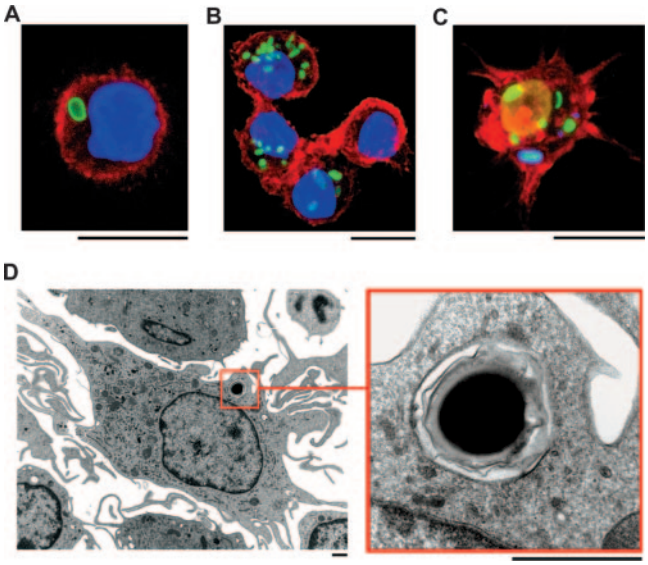

FIGURE 1. DCs endocytose *B. anthracis* spores. DCs were infected with an MOI of 10 Ames (*A*) or Sterne (*B*) spores (green) for 1 h, fixed, and stained for CD11c (red) and DNA (blue). *C*, Alternatively, DCs were stained for actin (red), DNA (gold), and germinated spores (blue) before analysis by confocal microscopy. *D*, DCs were infected for 15 min with Sterne spores, fixed, and analyzed by TEM (*left*). *Inset* of an internalized non-germinated spore is shown (*right*). Size bars indicate 10 (*A–C*) and 1 (*D*) μm.

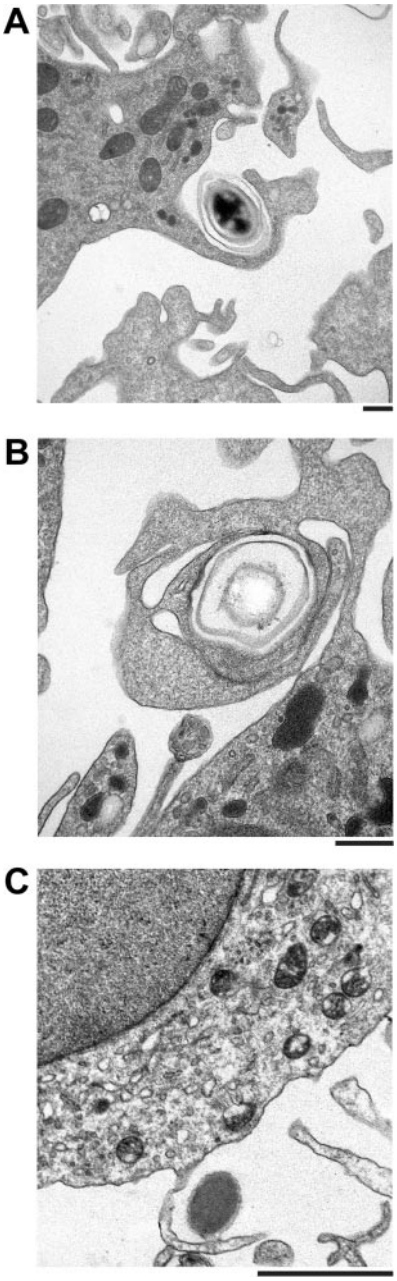

FIGURE 2. DCs engulf Sterne spores by coiling phagocytosis. DCs were infected 15 min with Sterne spores or latex beads, fixed, and analyzed by TEM. DCs endocytosed spores by macropinocytosis (*A*) and coiling phagocytosis (*B*). In contrast, DCs engulfed latex beads by macropinocytosis only (*C*). Size bars indicate 500 nm (*A* and *B*) and 2 μm (*C*).

Results

DC endocytosis of B. anthracis *spores*

To investigate whether DCs might play a role in *B. anthracis* pathogenesis, first we determined whether DCs internalize *B. anthracis* spores. Immature DCs derived from human monocytes were incubated with fully virulent Ames (pX01$^+$, pX02$^+$) spores for 1 h, and then rinsed to remove excess spores. The fixed cultures were labeled with Abs to the DC marker CD11c and to *B. anthracis* spores before examination by confocal microscopy. Ames spores were located inside CD11c$^+$ cells (Fig. 1*A*). Likewise, DCs internalized GFP-Sterne (pX01$^+$, pX02$^-$) spores (Fig. 1*B*) and GFP-ΔANR (pX01$^-$, pX02$^-$) spores (data not shown). Sterne and ΔANR spores that had clearly germinated based on staining of DNA were also found within DCs, although it was uncertain whether these spores were internalized after germination in the culture medium or had germinated within the DCs. Lung fluids alone are not sufficient to promote germination of inhaled spores (4), so phagocytic cells most likely engulf spores in a dormant state. Therefore, we examined the capacity for immature DCs to internalize ungerminated spores. After 1 h infection, fixed DCs were incubated with an Ab specific for *B. anthracis* bacilli to identify germinated spores (Fig. 1*C*). DCs were found to have internalized nongerminated GFP spores as indicated by absence of Ab binding. These data were also supported by TEM analysis of DCs that were infected for 15 min (Fig. 1*D*). An absence of swelling or elongation in the core region of internalized spores indicated they had not germinated.

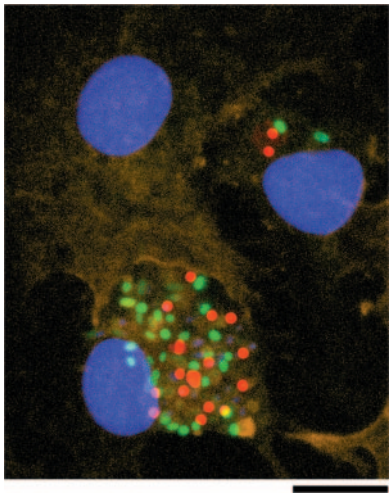

FIGURE 3. DCs show no preference for endocytosis between *B. anthracis* spores and latex beads. DCs were coincubated with premixed GFP spores and red latex beads in equal numbers (20 particles per DC total). DCs were stained for actin (gold) and DNA (blue). Size bar indicates 10 μm.

To determine the method of spore uptake, DCs were infected for 15 min and analyzed by TEM. Macropinocytosis was occasionally observed (Fig. 2*A*), but zipper-type phagocytosis was not. The predominant method used by DCs to take up spores was coiling phagocytosis (Fig. 2*B*). Among ~100 grids that showed spores

Table I. *DCs show no preference in endocytosis of Sterne (pX01$^+$) and ΔANR (pX01$^-$) spores*[a]

	% DCs Taking Up Spores (SD)[b]		Mean of Spores/DC (range)[c]	
MOI	Sterne	ΔANR	Sterne	ΔANR
1	5.8 (0.5)	4.6 (0.5)	1.0 (1)	1.8 (1–2)
5	12.5 (0.5)	10.4 (0.5)	1.6 (1–4)[d]	1.7 (1–7)[d]
10	20.4 (0.5)[d]	20.6 (0.4)[d]	2.8 (1–13)[e]	2.3 (1–9)[e]
50	30.6 (0.4)[d,e]	34.2 (0.4)[d,e]	3.7 (1–40)[f]	3.5 (1–20)[f]

[a] DCs were infected with GFP-Sterne or GFP-ΔANR spores for 1 h and gently fixed. For enumerating ingested spores, those adhered to the outside surface of DCs were distinguished from spores located inside of DCs by incubating nonpermeabilized cells with a mix of Abs specific for *B. anthracis* spores and bacilli, followed by a secondary incubation with Ab conjugated to Alexa-568-nm fluorophore. Approximately 100 DCs per sample were examined for enumerating internalized spores. These data are representative of responses from two different donors.

[b] Cochran-Armitage test (38) was used for statistical analysis of differences between MOI.

[c] Jonckheere-Terpstra test (39) was used for statistical analysis of differences between MOI.

[d] Significantly different from MOI of 1, $p < 0.040$.

[e] Significantly different from MOI of 5, $p < 0.020$.

[f] Significantly different from MOI of 10, $p = 0.004$.

associated with DCs, the mechanism of uptake was apparent in 30 grids. Of those, coiling phagocytosis was observed approximately two-thirds of the time. In contrast to spores, latex beads were engulfed by macropinocytosis without any evidence of coiling phagocytosis (Fig. 2*C*). These data indicate that the coiling mechanism to capture spores was neither a generalized response to particles of that size and shape nor a result of the culture conditions (16) used to generate DCs in vitro.

The amount of spore uptake varied widely from one cell to another, but examination of uptake during simultaneous incubation with spores and fluorescent latex beads suggested that differences in the extent of internalization were not specific to spores (Fig. 3). Although incubation with increasing numbers of spores led to significantly greater numbers of DCs taking up spores on average (Table I), considerable variation among DCs persisted, including some cells that exhibited no uptake (Table I). Because the level of maturation is known to influence DC endocytic activity (17), it is likely that this observation reflects heterogeneity in maturity of cultured DCs.

DC chemokine receptor expression, maturation, and enhanced costimulation following infection with B. anthracis *spores*

Having confirmed that DCs capture *B. anthracis* spores, we examined the subsequent impact on DCs to determine whether spores promote lymph node homing and maturation. During an infection, anthrax toxins might influence DC function, so we compared responses to two different strains of spores: Sterne strain ($pX01^+$) and ΔANR strain ($pX01^-$). We had already confirmed that there was no apparent difference in quantities of internalized spores using these two *B. anthracis* strains (Table I).

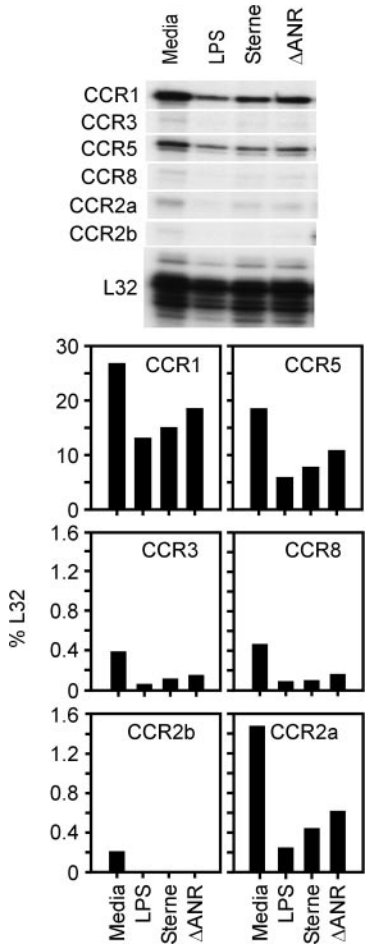

FIGURE 4. *B. anthracis* spores induce loss of tissue-retaining chemokine receptor RNA. RPA template probe set hCR5 was hybridized with RNA from DCs treated with 100 ng/ml LPS, Sterne spores, or ΔANR spores for 2 h. Band density of chemokine receptor-specific mRNA-protected probe fragments (*top*) was expressed as a percentage of L32 (*bottom*). These data are representative of responses from three different donors.

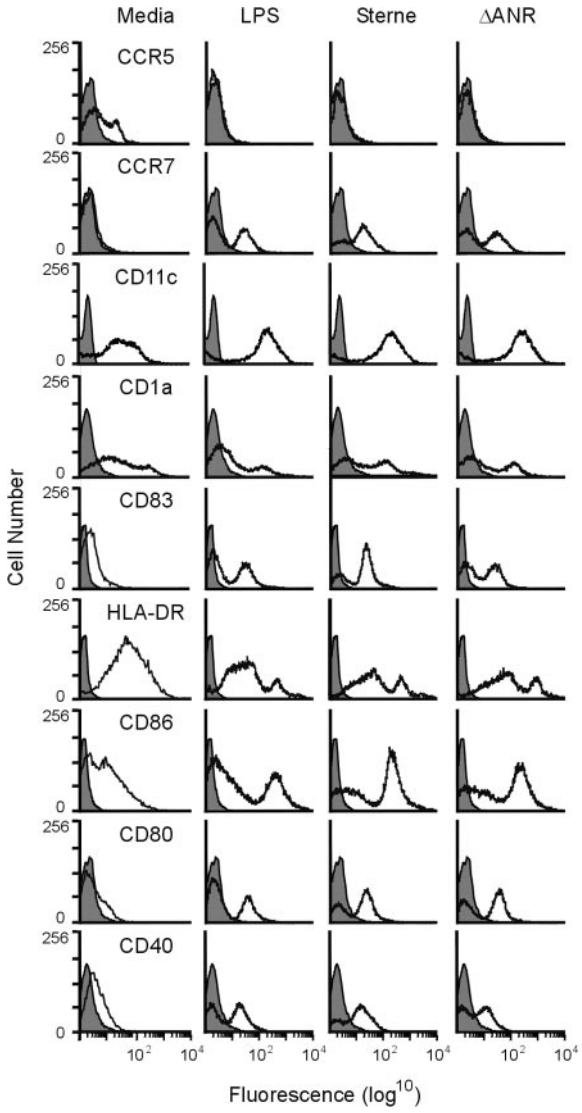

FIGURE 5. DCs infected with *B. anthracis* spores mature and develop the capacity to migrate into lymph nodes. DCs were treated with 100 ng/ml LPS, Sterne spores, or ΔANR spores for 2 h before antibiotic was added. Following 24 h incubation, cell surface protein expression was analyzed by flow cytometry using protein-specific Abs (black line) or isotype control IgG (gray filled). These data are representative of responses from six different donors.

DCs were treated with Sterne spores, ΔANR spores, or the standard activating agent bacterial LPS for 2 h before harvesting samples. RPA revealed that expression of RNA for tissue-retaining chemokine receptors (CCR1, CCR3, CCR5, CCR8, and CCR2ab) was reduced to a similar extent by treatment with Sterne spores, ΔANR spores, and LPS (Fig. 4). These changes are characteristic of lymph node homing DCs. For cell surface analysis of protein expression, DCs were treated with LPS or infected with Sterne or ΔANR spores for 2 h before gentamicin was added to the culture medium and incubated 22 h longer. Flow cytometric analysis (Fig. 5) revealed that all three treatments increased expression of CCR7,

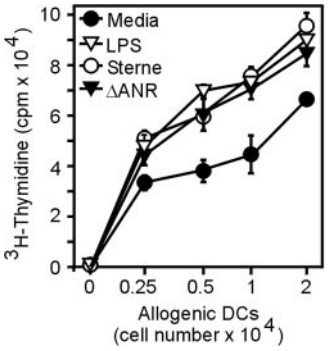

FIGURE 6. Spore-infected DCs develop enhanced allostimulatory function. DCs were treated with medium (●), 100 ng/ml LPS (▽), Sterne spores (○), or ΔANR spores (▼) for 6 h before cultures were irradiated and incubated at the indicated numbers with 1×10^5 allogenic T cells. [^{3}H]Thymidine incorporation (cpm $\times 10^4$) by proliferating T cells is displayed as the mean of triplicate determinations. These data are representative of responses from four different donors.

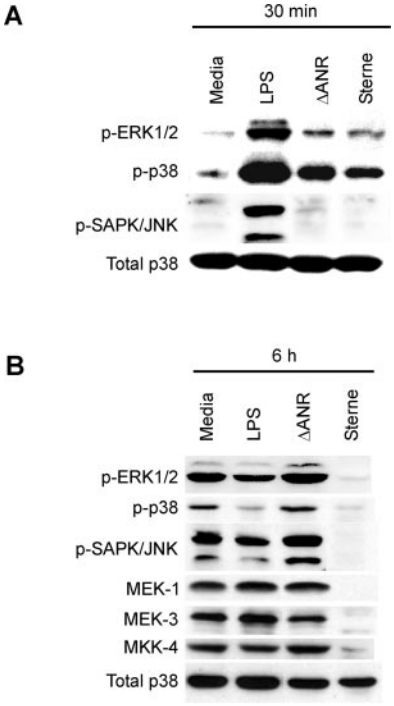

FIGURE 7. *B. anthracis* virulence factors deactivate proinflammatory responses initiated by spores. DCs were treated as indicated for 30 min (*A*) or 6 h (*B*) before samples were analyzed by Western blot using protein and phosphoprotein-specific Abs. These data are representative of responses from four different donors.

a chemokine receptor required for DCs to migrate into lymphatic vessels (18) and to T cell zones in the lymph node (19). Like LPS, spores increased the mean fluorescent intensity of CD11c$^+$ DCs, and there was a loss of CCR5 at the membrane surface (Fig. 5). These changes also facilitate migration to lymph nodes. The maturation marker CD83 and proteins that enable efficient activation of cognate T cells (HLA-DR, CD40, CD80, and CD86) were expressed at higher levels (increased mean fluorescent intensity and percentage of positive DCs), whereas CD1a levels were reduced at the membrane surface of spore- and LPS-treated DCs (Fig. 5). These experiments were repeated exactly as before, except gentamicin was added 6 h after infection, and yielded results similar to those shown. These phenotypic changes indicate that spores induce maturation of DCs.

To examine whether spore infection impaired functional maturation, the capacity for DCs to activate T cells was examined by incorporation of [^{3}H]thymidine by proliferating cells (Fig. 6). DCs were infected with spores for 6 h before the cultures were irradiated and incubated with purified allogenic T cells. Statistical analysis by *t* test indicated significantly higher levels of [^{3}H]thymidine incorporation when DCs were treated with Sterne ($p = 0.01$ at 1×10^4, $p = 0.02$ at 2×10^4) and ΔANR ($p = 0.03$ at 1×10^4, $p = 0.05$ at 2×10^4) than DCs treated with medium alone. The loss of tissue-retaining chemokine receptors, de novo expression of lymph node homing, and costimulatory proteins, as well as enhanced costimulatory function indicate that spore-infected DCs mature and develop the capacity to migrate into draining lymph nodes. These changes could facilitate dissemination of *B. anthracis* throughout the host.

B. anthracis *spores activate MAPK signaling in DCs*

The *B. anthracis* toxins are important factors that can influence proinflammatory activity of mononuclear cells when added as purified proteins (20, 21). Spores stimulated DC maturation and the capacity to migrate into lymph nodes. To our surprise, there was no apparent influence of anthrax toxins on these responses to spore infection (Fig. 5). Therefore, the next question we asked was whether anthrax toxins might facilitate immune evasion by inhibiting proinflammatory responsiveness of DCs. We first tested for activation of the MAPK cascade, which regulates expression of many inflammatory response genes in DCs (22). DCs were treated with LPS, Sterne, ΔANR, or inactivated Sterne spores, and cellular proteins were harvested for Western analysis of activated (i.e., phosphorylated) MAPKs. Within 30 min of infection, ΔANR spores and Sterne spores induced weak phosphorylation of ERK1/2 and strong phosphorylation of p38 (Fig. 7*A*), whereas LPS induced strong phosphorylation of ERK1/2 and p38. Like the live spores, inactivated Sterne spores induced a similar pattern of phosphorylation of ERK and p38 (data not shown), suggesting that the spore coat contains molecules capable of activating DCs. Unlike LPS, Sterne and ΔANR spores did not activate stress-activated protein kinase (SAPK)/JNK within 30 min of infection (Fig. 7*A*). DCs treated with ΔANR spores showed weak phosphorylation of ERK1/2 and p38 as well as SAPK/JNK phosphorylation at 6 h (Fig. 7*B*), but Sterne-treated DC cultures lacked any detectable phosphorylation. Activation of the MAPKs as long as 6 h may result from ΔANR bacilli present in abundance after 2 h of infection and from a presence of nongerminated spores.

The capability of Sterne, but not ΔANR bacilli to produce LT suggested that LT may inactivate the MAPKs via cleavage of kinases (MEKs or MAP II kinase kinases (MKKs) immediately upstream of MAPKs (20, 23–25). Therefore, we examined levels of MEKs that phosphorylate ERK1/2 (MEK-1), p38 (MEK-3), and SAPK/JNK (MKK-4) at 6 h after infection. Protein samples were probed using Abs that detect peptide sequences of MEK-1, MEK-3, and MKK-4 that are detached by enzymatic activity of LT. Partial cleavage of all three proteins was evident by 4 h in Sterne-treated DCs, but not in ΔANR- or LPS-treated samples (data not shown). Complete cleavage of all MEK-1 and MEK-3 protein and nearly complete cleavage of all MKK-4 protein were observed at 6 h postinfection (Fig. 7*B*). These data indicate that spores initially activated DCs. As virulence factors such as LT reach a threshold, the toxins silence transduction of further intracellular signals, effectively shutting down the MAPK pathway.

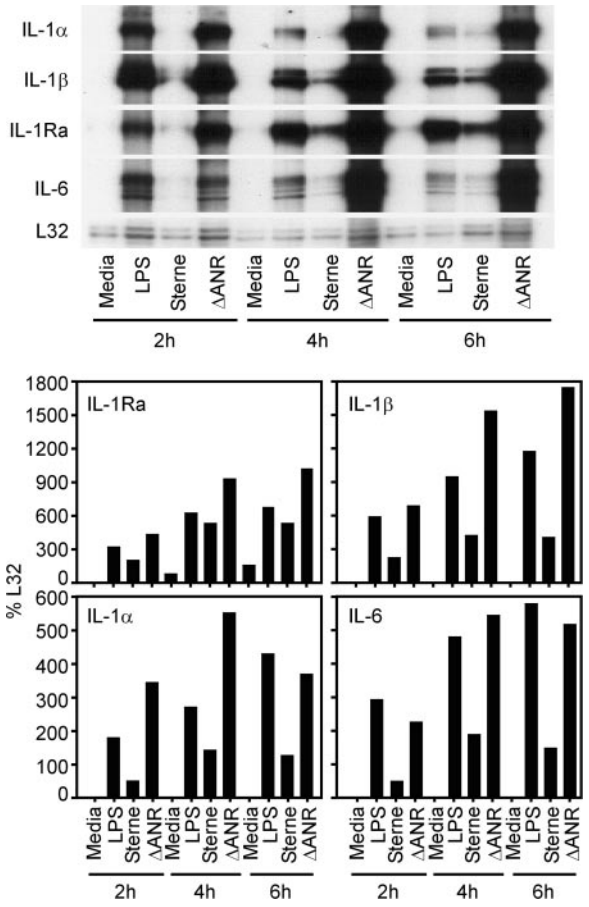

FIGURE 8. Spore-infected DCs produce inflammatory response gene mRNA. RPA template set hCK2b was hybridized with RNA from DCs treated with 100 ng/ml LPS, Sterne spores, or ΔANR spores for the indicated times. Band density of cytokine-specific mRNA-protected probe fragments (*top*) was expressed as a percentage of L32 (*bottom*). These data are representative of responses from three different donors.

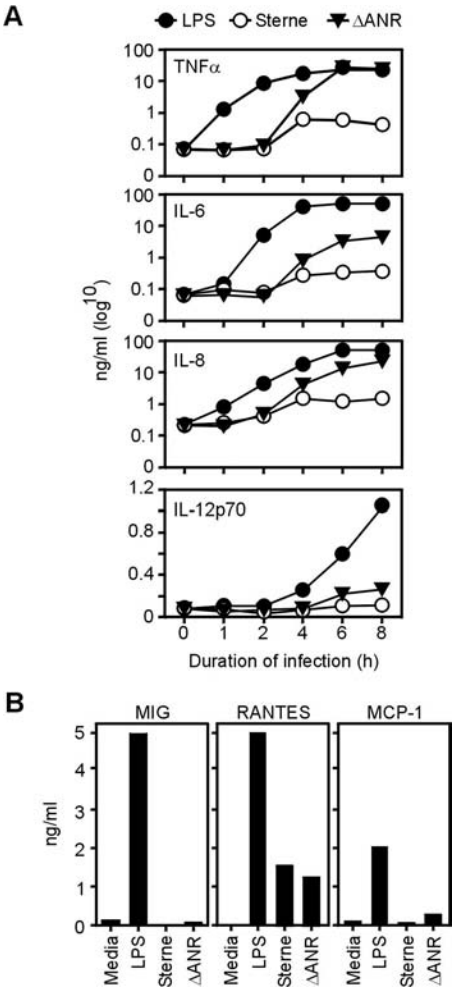

FIGURE 9. *B. anthracis* spore infection stimulates secretion of proinflammatory mediators. Cytometric bead array analysis was performed on culture supernatants harvested after treatment for the indicated times (*A*) with 100 ng/ml LPS (●), Sterne spores (○), or ΔANR spores (▼). Concentrations of secreted proteins are shown on a logarithmic scale in *A*. *B*, DCs were treated as in *A* for 4 h. These data are representative of responses from six different donors.

Inflammatory response gene expression from DCs infected with B. anthracis *spores*

Termination of MAPK signaling would be expected to inhibit expression of inflammatory response genes and curtail secretion of proinflammatory cytokines and chemokines, which are critical for activating the innate immune system. To assess this, we examined expression from genes regulated by the MAPK signaling pathway such as TNF-α, IL-6, IL-1β, MCP-1, IL-8, as well as other mediators of inflammation (22). RPA revealed that IL-1α, IL-1β, IL-1Ra, and IL-6 mRNA were induced by treatment with LPS and ΔANR spores at similar levels, and by Sterne spores to a lesser extent, by 2 h (Fig. 8). In LPS and ΔANR spore-treated samples, the IL-1α, IL-1β, IL-1Ra, and IL-6 mRNA levels continued to increase by 4 h. By 6 h, LPS and ΔANR samples continued to show increasing levels of IL-1β and IL-1Ra mRNA. In contrast, Sterne-treated samples showed a uniform plateau in mRNA levels after 4-h infection.

Secreted cytokines and chemokines were examined by cytometric bead array using culture supernatants harvested from DCs treated with Sterne spores, ΔANR spores, or LPS. LPS and ΔANR spores stimulated secretion of TNF-α, IL-6, and IL-8 in copious amounts that continued to rise at 8 h posttreatment (Fig. 9*A*). In contrast, DCs infected with Sterne spores secreted overall lower levels of these inflammatory mediators and did not continue to rise after 4-h infection. Other mediators of inflammation such as IL-12p70 (Fig. 9*A*) were stimulated by LPS, but not by Sterne or ΔANR spores, suggesting differential activation of specific genes. Surprisingly, both Sterne and ΔANR spores poorly stimulated secretion of other chemokines. From spore-treated DCs, monokine induced by IFN-γ was not secreted, and only negligible levels of MCP-1 and moderate levels of RANTES were observed 4 h after infection (Fig. 9*B*). Examination of DCs by trypan blue exclusion (data not shown) indicated that infection did not induce DC death by 8 h after infection. These data demonstrate that human DCs respond to infection with *B. anthracis* spores by producing RNA and secreting inflammatory response proteins, which modulate inflammation and promote maturation of DCs. Furthermore, *B. anthracis* virulence factors, most likely those encoded on the pX01 plasmid, later suppress secretion of inflammatory response proteins in part by impairing transduction of signals through the MAPK cascade.

Discussion

Although DCs are recognized as important sentinels of microbial invasion in the lungs, recent studies on *Aspergillus fumigatus* and

Mycobacterium tuberculosis have suggested that DCs may sometimes contribute to disease pathogenesis by activating a nonprotective Th2 response or by serving as a hidden reservoir for bacteria (26–28). Our results suggest that this might be true for infection with *B. anthracis* spores, which may possibly convert DCs into a vehicle for dissemination. In vivo testing of this hypothesis in an appropriate animal model will be necessary to determine whether DCs disseminate spores during infection in vivo.

Our data clearly demonstrate that immature human DCs are capable of internalizing nongerminated *B. anthracis* spores. Interestingly, DCs engulf spores primarily by means of coiling phagocytosis, a receptor-mediated (29) method predominantly associated with uptake of pathogenic organisms. Coiling phagocytosis is a disorganized form of zipper-type phagocytosis characterized by single folds of plasma membrane that wrap around a microbe in multiple turns (30). Although the significance of coiling phagocytosis for disease pathogenesis has yet to be elucidated, it is a subject that surely warrants further investigation.

B. anthracis might induce its exit out of the lungs within DCs, in part, by activating the MAPK signaling cascade. In our system, we observed weak activation of ERK with strong activation of p38 (Fig. 7) quickly after endocytosis of spores. In DCs, this pattern of MAPK activation stimulates lymph node homing via secretion of proinflammatory cytokines (e.g., TNF-α) known to enable DC trafficking between the lungs and mediastinal lymph nodes (31–33). Although greatly attenuated in the presence of *B. anthracis* with preserved pX01 virulence factors, cytokine secretion was apparently sufficient to stimulate pathways that promoted maturation, enhanced activation of allogenic T cells, and membrane protein changes essential for DC migration into lymph nodes.

An effect of virulence factors, such as LT, was evident by 6 h postinfection when suppression of MAPK signaling and production of proteins from downstream target genes were observed. In a previous study, Agrawal et al. (34) reported impaired phosphorylation of ERK1/2 and p38 in response to LPS and a reduction in cytokine expression after 24 h preincubation of murine DCs with purified LT. Our data suggest that the levels of LT released by Sterne bacilli are sufficient to achieve these same effects, in part, by cleavage of MEK-1, MEK-3, and MKK-4. Significantly, our study agrees with their MAPK signaling results using a different experimental system, which involved spore infection with permitted outgrowth of *B. anthracis* bacilli and production of virulence factors. Our study and that of Agrawal et al. (34) contrast with a new study (35) that showed no impact of Sterne infection on cytokine secretion. Although our results did not indicate an impact of specific virulence factors on expression of the costimulatory membrane proteins that we examined, Agrawal et al. (34) reported a suppression of costimulatory molecule expression with overnight LT preincubation and subsequent LPS stimulation. Significantly, these differences could be reconciled by the capacity for spores to stimulate proinflammatory pathways before activity of virulence factors. Also, new immunostimulatory bacterial products may have become accessible to DCs during incubation with gentamicin, and thus provide further stimulation for DCs to mature.

Because Sterne bacilli are capable of producing both ET and LT, there may be a combined effect of these exotoxins on cytokine and chemokine production. Edema factor is a calmodulin-activated adenylate cyclase (36), which binds to heptamers of protective Ag to traverse cell membranes, forming ET. By increasing intracellular cAMP levels, ET inhibits TNF-α and IL-6 secretion from monocytes (21). To our knowledge, no studies have investigated the effects of ET on DCs. However, it was shown that elevated cAMP levels can inhibit release of proinflammatory cytokines without affecting the differentiation process of DCs (37).

Overall, our study demonstrates that *B. anthracis* virulence factors suppress proinflammatory responsiveness of DCs. Because DCs are regarded as a critical link between the innate and adaptive immune responses (10), impaired responsiveness may diminish DC-mediated activation of other leukocytes in the innate immune system and facilitate escape of *B. anthracis* from detection, thereby enhancing its ability to complete the infection process.

The results of this study are entirely consistent with a hypothesized scenario in which *B. anthracis* spores may use DCs to facilitate infection. The sequence of events would be initiated by spores stimulating their endocytosis by DCs. DCs would then become transiently activated in a proinflammatory mode and secrete inflammatory cytokines, undergo maturation, and migrate to draining lymph nodes. Further production of anthrax toxins by bacilli would silence the proinflammatory response and delay an immune response to the infection. Although additional work will be required to confirm this sequence of events, the current study provides a sound foundation of plausibility.

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Disclosures

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References

1. Guarner, J., J. A. Jernigan, W. J. Shieh, K. Tatti, L. M. Flannagan, D. S. Stephens, T. Popovic, D. A. Ashford, B. A. Perkins, and S. R. Zaki. 2003. Pathology and pathogenesis of bioterrorism-related inhalational anthrax. *Am. J. Pathol. 163:701*.
2. Reissman, D. B., E. A. Whitney, T. H. Taylor, Jr., J. A. Hayslett, P. M. Dull, I. Arias, D. A. Ashford, E. A. Bresnitz, C. Tan, N. Rosenstein, and B. A. Perkins. 2004. One-year health assessment of adult survivors of *Bacillus anthracis* infection. *J. Am. Med. Assoc. 291:1994*.
3. Guidi-Rontani, C., M. Weber-Levy, E. Labruyere, and M. Mock. 1999. Germination of *Bacillus anthracis* spores within alveolar macrophages. *Mol. Microbiol. 31:9*.
4. Ross, J. M. 1957. The pathogenesis of anthrax following the administration of spores by the respiratory route. *J. Pathol. Bacteriol. 78:485*.
5. Lincoln, R. E., D. R. Hodges, F. Klein, B. G. Mahlandt, W. I. Jones, Jr., B. W. Haines, M. A. Rhian, and J. S. Walker. 1965. Role of the lymphatics in the pathogenesis of anthrax. *J. Infect. Dis. 115:481*.
6. Shafazand, S., R. Doyle, S. Ruoss, A. Weinacker, and T. A. Raffin. 1999. Inhalational anthrax: epidemiology, diagnosis, and management. *Chest 116:1369*.
7. Guidi-Rontani, C. 2002. The alveolar macrophage: the Trojan horse of *Bacillus anthracis*. *Trends Microbiol. 10:405*.
8. Cote, C. K., K. M. Rea, S. L. Norris, R. N. Van, and S. L. Welkos. 2004. The use of a model of in vivo macrophage depletion to study the role of macrophages during infection with *Bacillus anthracis* spores. *Microb. Pathog. 37:169*.
9. Stumbles, P. A., J. W. Upham, and P. G. Holt. 2003. Airway dendritic cells: co-ordinators of immunological homeostasis and immunity in the respiratory tract. *APMIS 111:741*.
10. Steinman, R. M. 2001. Dendritic cells and the control of immunity: enhancing the efficiency of antigen presentation. *Mt. Sinai J. Med. 68:106*.
11. Bosio, C. M., M. J. Aman, C. Grogan, R. Hogan, G. Ruthel, D. Negley, M. Mohamadzadeh, S. Bavari, and A. Schmaljohn. 2003. Ebola and marburg viruses replicate in monocyte-derived dendritic cells without inducing the production of cytokines and full maturation. *J. Infect. Dis. 188:1630*.
12. Ruthel, G., W. J. Ribot, S. Bavari, and T. A. Hoover. 2004. Time-lapse confocal imaging of development of *Bacillus anthracis* in macrophages. *J. Infect. Dis. 189:1313*.
13. Millonig, G. 1961. Advantages of a phosphate buffer for OsO4 solutions in fixation. *J. Appl. Physiol. 32:1637*.
14. Moayeri, M., D. Haines, H. A. Young, and S. H. Leppla. 2003. *Bacillus anthracis* lethal toxin induces TNF-α-independent hypoxia-mediated toxicity in mice. *J. Clin. Invest. 112:670*.
15. Thellin, O., W. Zorzi, B. Lakaye, B. B. De, B. Coumans, G. Hennen, T. Grisar, A. Igout, and E. Heinen. 1999. Housekeeping genes as internal standards: use and limits. *J. Biotechnol. 75:291*.
16. Rittig, M. G., J. C. Jagoda, B. Wilske, R. Murgia, M. Cinco, R. Repp, G. R. Burmester, and A. Krause. 1998. Coiling phagocytosis discriminates between different spirochetes and is enhanced by phorbol myristate acetate and granulocyte-macrophage colony-stimulating factor. *Infect. Immun. 66:627*.
17. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature 392:245*.
18. Gunn, M. D., K. Tangemann, C. Tam, J. G. Cyster, S. D. Rosen, and L. T. Williams. 1998. A chemokine expressed in lymphoid high endothelial

venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proc. Natl. Acad. Sci. USA 95:258*.
19. Ngo, V. N., H. L. Tang, and J. G. Cyster. 1998. Epstein-Barr virus-induced molecule 1 ligand chemokine is expressed by dendritic cells in lymphoid tissues and strongly attracts naive T cells and activated B cells. *J. Exp. Med. 188:181*.
20. Pellizzari, R., C. Guidi-Rotani, G. Vitale, M. Mock, and C. Montecucco. 1999. Anthrax lethal factor cleaves MKK3 in macrophages and inhibits the LPS/IFNγ-induced release of NO and TNFα. *FEBS Lett. 462:199*.
21. Hoover, D. L., A. M. Friedlander, L. C. Rogers, I. K. Yoon, R. L. Warren, and A. S. Cross. 1994. Anthrax edema toxin differentially regulates lipopolysaccharide-induced monocyte production of tumor necrosis factor α and interleukin-6 by increasing intracellular cyclic AMP. *Infect. Immun. 62:4432*.
22. Saccani, S., S. Pantano, and G. Natoli. 2002. p38-Dependent marking of inflammatory genes for increased NF-κB recruitment. *Nat. Immunol. 3:69*.
23. Duesbery, N. S., C. P. Webb, S. H. Leppla, V. M. Gordon, K. R. Klimpel, T. D. Copeland, N. G. Ahn, M. K. Oskarsson, K. Fukasawa, K. D. Paull, and G. F. Vande Woude. 1998. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. *Science 280:734*.
24. Vitale, G., R. Pellizzari, C. Recchi, G. Napolitani, M. Mock, and C. Montecucco. 1998. Anthrax lethal factor cleaves the N-terminus of MAPKKs and induces tyrosine/threonine phosphorylation of MAPKs in cultured macrophages. *Biochem. Biophys. Res. Commun. 248:706*.
25. Turk, B. E., T. Y. Wong, R. Schwarzenbacher, E. T. Jarrell, S. H. Leppla, R. J. Collier, R. C. Liddington, and L. C. Cantley. 2004. The structural basis for substrate and inhibitor selectivity of the anthrax lethal factor. *Nat. Struct. Mol. Biol. 11:60*.
26. Bodnar, K. A., N. V. Serbina, and J. I. Flynn. 2001. Fate of *Mycobacterium tuberculosis* within murine dendritic cells. *Infect. Immun. 69:800*.
27. Bozza, S., R. Gaziano, A. Spreca, A. Bacci, C. Montagnoli, P. di Francesco, and L. Romani. 2002. Dendritic cells transport conidia and hyphae of *Aspergillus fumigatus* from the airways to the draining lymph nodes and initiate disparate Th responses to the fungus. *J. Immunol. 168:1362*.
28. Jiao, X., R. Lo-Man, P. Guermonprez, L. Fiette, E. Deriaud, S. Burgaud, B. Gicquel, N. Winter, and C. Leclerc. 2002. Dendritic cells are host cells for mycobacteria in vivo that trigger innate and acquired immunity. *J. Immunol. 168:1294*.
29. Horwitz, M. A. 1984. Phagocytosis of the Legionnaires' disease bacterium (*Legionella pneumophila*) occurs by a novel mechanism: engulfment within a pseudopod coil. *Cell 36:27*.
30. Rittig, M. G., G. R. Burmester, and A. Krause. 1998. Coiling phagocytosis: when the zipper jams, the cup is deformed. *Trends Microbiol. 6:384*.
31. Cumberbatch, M., and I. Kimber. 1992. Dermal tumor necrosis factor-α induces dendritic cell migration to draining lymph nodes, and possibly provides one stimulus for Langerhans' cell migration. *Immunology 75:257*.
32. Luft, T., E. Maraskovsky, M. Schnurr, K. Knebel, M. Kirsch, M. Gorner, R. Skoda, A. D. Ho, P. Nawroth, and A. Bierhaus. 2004. Tuning the volume of the immune response: strength and persistence of stimulation determine migration and cytokine secretion of dendritic cells. *Blood 104:1066*.
33. Roake, J. A., A. S. Rao, P. J. Morris, C. P. Larsen, D. F. Hankins, and J. M. Austyn. 1995. Dendritic cell loss from non-lymphoid tissues after systemic administration of lipopolysaccharide, tumor necrosis factor, and interleukin 1. *J. Exp. Med. 181:2237*.
34. Agrawal, A., J. Lingappa, S. H. Leppla, S. Agrawal, A. Jabbar, C. Quinn, and B. Pulendran. 2003. Impairment of dendritic cells and adaptive immunity by anthrax lethal toxin. *Nature 424:329*.
35. Pickering, A. K., M. Osorio, G. M. Lee, V. K. Grippe, M. Bray, and T. J. Merkel. 2004. Cytokine response to infection with *Bacillus anthracis* spores. *Infect. Immun. 72:6382*.
36. Leppla, S. H. 1982. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. *Proc. Natl. Acad. Sci. USA 79:3162*.
37. Galgani, M., V. De Rosa, S. De Simone, A. Leonardi, U. D'Oro, G. Napolitani, A. M. Masci, S. Zappacosta, and L. Racioppi. 2004. Cyclic AMP modulates the functional plasticity of immature dendritic cells by inhibiting Src-like kinases through protein kinase A-mediated signaling. *J. Biol. Chem. 279:32507*.
38. Agresti, A. 2002. *Categorical Data Analysis*. John Wiley & Sons, Hoboken, p. 181.
39. Hollander, M., and D. A. Wolfe. 1999. *Nonparametric Statistical Methods*. John Wiley & Sons, New York, p. 202.